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The effect of elevated temperature on gene transcription and aflatoxin biosynthesis

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Abstract: The molecular regulation of aflatoxin biosynthesis is complex and influenced by several environmental conditions; one of these is temperature. Aflatoxins are produced optimally at 28–30 C, and production decreases as temperatures approach 37 C, the optimum temperature for fungal growth. To better characterize the influence of temperature on aflatoxin biosynthesis, we monitored the accumulation of aflatoxin and the expression of more than 5000 genes in *Aspergillus flavus* at 28 C and 37 C. A total of 144 genes were expressed differentially ($P < 0.001$) between the two temperatures. Among the 103 genes more highly expressed at 28 C, approximately 25% were involved in secondary metabolism and about 30% were classified as hypothetical. Genes encoding a catalase and superoxide dismutase were among those more highly expressed at 37 C. As anticipated we also found that all the aflatoxin biosynthetic genes were much more highly expressed at 28 C relative to 37 C. To our surprise expression of the pathway regulatory genes *aflR* and *aflS*, as well as *aflR* antisense, did not differ between the two temperatures. These data indicate that the failure of *A. flavus* to produce aflatoxin at 37 C is not due to lack of transcription of *aflR* or *aflS*. One explanation is that AFLR is nonfunctional at high temperatures. Regardless, the factor(s) sensing the elevated temperatures must be acute. When aflatoxin-producing cultures are transferred to 37 C they immediately stop producing aflatoxin.

Key words: AFLR, biosynthesis, micro-arrays

INTRODUCTION

Aflatoxin is a highly carcinogenic polyketide secondary metabolite produced by several species of Aspergilli, including *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. bombycis*, (Samson 2001, Varga et al 2003) and others (Cary et al 2005). *Aspergillus flavus* along with *A. parasiticus* is a well known pathogen of many economically important commodities including corn, peanuts, cotton and tree nuts. Infection of seeds and contamination with aflatoxin is not limited to the field but also can occur postharvest if seeds are stored improperly. Exposure to aflatoxins has been associated with liver cancer and many veterinary toxic syndromes (Bressac et al 1991, Hsu et al 1991, Wang et al 2001, Lewis et al 2005). The United States, along with many other developed nations, have imposed regulatory limits on aflatoxin in food and feed. In the US agricultural economic losses due to aflatoxin contamination of food and feed are estimated to be $270 million annually (Richard and Payne 2003). The regulation of aflatoxin biosynthesis is influenced by several environmental and cultural conditions such as temperature, pH, adenylate concentration and energy charge, and nitrogen and carbon source (Luchese and Harrigan 1993, Payne and Brown 1998, Price et al 2005). The influence of temperature on aflatoxin biosynthesis has intrigued researchers because only low concentrations of aflatoxin are produced at the temperatures that are optimal for fungal growth (37 C).

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Early studies by Schindler et al (Schindler et al 1967) showed that aflatoxin was produced maximally at 24 C and not at all at temperatures lower than 18 C or higher than 35 C. However Diener and Davis reported aflatoxin production in peanuts at 40 C by *A. flavus* (Diener and Davis 1967). Mayne et al (1967) suggested that the effect of temperature is more dependent on substrate than on strain. It also has been reported that the production of aflatoxin or its pathway intermediates are regulated differently among some aspergilli species. Feng and Leonard compared *A. parasiticus* to *A. nidulans* under varying culture conditions. They detected aflatoxin at 27 C and lesser amounts at 33 C but were unable to detect aflatoxin in *A. parasiticus* at 37 C. In contrast they found that *A. nidulans* produced sterigmatocystin at similar levels at all three temperatures (Feng and Leonard 1998). While the cardinal range for aflatoxin production differs among strains and culture conditions, most research shows temperatures between 24–30 C favor aflatoxin biosynthesis.

The mechanism underlying the temperature dependent regulation of aflatoxin production is unclear. Both transcriptional and posttranscriptional regulation mechanisms control aflatoxin gene transcription (for reviews see Payne and Brown 1998; Bennett and Klich 2003; Bhatnagar et al 2003; Yu et al 2004a, b). Feng and Leonard (1995) used northern analysis to show that the aflatoxin polyketide synthase gene of *A. parasiticus* was expressed at 27 C but not at 37 C. Another pathway gene, *aflP* (*omtA*) also was shown to be transcribed in *A. parasiticus* at 29 C but not 37 C (Liu and Chu 1998). Because both these genes are regulated by AFLR, Liu and Chu examined cultures of *A. parasiticus* grown at different temperatures for the presence of AFLR and *aflR* transcripts. Cultures were grown at 29 C or 37 C on PMS (a medium nonconductive for aflatoxin production) and subsequently transferred to GMS (a conducive medium for aflatoxin production). Transcripts of *aflR* and AFLR were present in cultures grown at both 29 C and 37 C, but the levels of AFLR were reduced fourfold at 37 C. In addition to *aflR*, another gene in the pathway, *aflS* has been shown to have a regulatory role in aflatoxin biosynthesis (Meyers et al 1998, Chang 2003). The AFLS protein binds to AFLR and modulates its expression.

More recently micro-arrays have been developed to evaluate gene transcription during aflatoxin biosynthesis (OBrian et al 2003; Price et al 2005, 2006). The availability of DNA micro-arrays of *A. flavus* containing more than 5000 elements from an EST library (Yu et al 2004c) provides the opportunity to better examine the effect of temperature on the pathway regulatory genes as well as nearly half of the genes in the *A. flavus* genome (www.aspergillusflavus.org).

MATERIAL AND METHODS

Growth, media and aflatoxin analysis.—Aspergillus flavus strain NRRL 3357 (ATCC 200026; SRRC 167), a wild type *A. flavus* strain widely used in laboratory and field studies, as well as the strain of choice for the whole genome sequencing project, was used for gene transcription analysis and growth studies. For gene transcription and temperature shift experiments, a mother culture supplemented with 0.4% agar was seeded with 1×10^6 spores/mL. Mother cultures were grown in A&M media at 37 C and 200 rpm for 16 h, and a 20 mL aliquot was used to inoculate 200 mL of fresh A&M media. Daughter cultures were grown at 28 C, 35 C or 37 C and shaken at 200 rpm. Aflatoxin concentrations were determined as indicated either by HPLC or LC/MS at the Proteomics and Metabolomics Center (N.C. State University).

To determine the effect of temperature on aflatoxin production in liquid grown cultures, 1×10^6 spores/mL were seeded into 100 mL A&M media and incubated at selected temperatures. For similar studies performed on solid media, fresh spores were generated by plating 50 µL of 10^8 spores onto Difco potato-dextrose agar (PDA) (American Scientific Products, Charlotte, North Carolina) and incubated at 30 C for 5 d. The spores were collected from 5 d cultures with sterile 0.05% Triton X-100. Samples were grown and collected according to Abbas et al (2004) with minor modifications by plating 100 µL of 107 spores of each isolate on PDA enriched with 0.3% β-CD (CD-PDA) (Cavasol®W7M, Wacker-Chemie GmbH, Burghausen, Germany). Duplicate cultures of each isolate were incubated for 24 h in total darkness at 28 C, 29 C, 30 C, 31 C, 32 C, 33 C, 34 C, 35 C, 36 C and 37 C. Fungal biomass and agar were removed from each sample with an inverted 1 mL pipette tip placed in glass scintillation vials (20 mL) and fresh weights were recorded (typically 0.5–1.0 g). A 10 : 1 volume of methanol-water (70 : 30, v/v) was added to the samples and the vials were shaken for 1 h at low speed on a reciprocal shaker. A 1 mL aliquot of extract was removed and centrifuged at 12 000 *g* for 10 min and the supernatant was assayed for the presence of aflatoxins, using ELISA kits (Veratox, Neogen Corp., Lansing, Michigan). Acetonitrile was mixed 1 : 1 with 500 µL of the extract, and 800 µL of this mixture was cleaned with an Alltech 1.5 mL Extract-Clean reservoir containing 200 mg of aluminium oxide. The extract was eluted by gravity and a total of 20 µL was examined by HPLC (Sobolev and Dorner 2002). Quantitation of aflatoxins was determined by the external standard method where the standard curve was 0.5–20 ng mL^{-1} (AFB1, AFG1) and 0.2–6 ng mL^{-1} (AFB2, AFG2) (Sigma).

RNA isolation.—For micro-array and QPCR experiments, RNA was isolated from lyophilized cultures with Trizol (Life Technologies, Rockville, Maryland) according to the manufacturer's instructions. Isolated RNA was purified further by precipitation on ice overnight in 2 M LiCl. The RNA was pelleted, washed with 70% ethanol and air-dried about 10 min. The RNA pellet was resuspended in 50 mL DEPC-dH_2O with 40 units RNasin™ RNase inhibitor (Promega Corporation, Madison, Wisconsin) and quantified by spectrophotometry.

QPCR.—RNA (1 μg) isolated from 28 C, 35 C and 37 C cultures grown 24 h was used in a reverse transcription reaction (Stratascript) to synthesize the cDNA template. QPCR reactions were performed in triplicate with a DNA Engine Opticon 2 System (MJ Research) and data were collected with Opticon Monitor Software version 2.02 (MJ Research). SYBR-green master mix (Applied Biosystems) was used to monitor expression with a 96-well format. Expression levels were measured in triplicate and calculated by a variation of comparative C(t) method (Livak and Schmittgen 2001) with 18s rRNA as the endogenous reference for sample normalization. For each set of temperatures, the mean of the normalized C(t) values for a given gene was used to measure fold increase relative to that gene across conditions tested. To provide a conservative estimate of the mean, the maximum delta C(t) score possible given the number of cycles run was assigned for samples where no expression was detected.

Micro-arrays.—Micro-arrays used in this study were printed at The Institute for Genome Research (TIGR) with amplicons (approx. 500 bp) from EST clones (Yu et al 2004c). A total of 5002 genes were arrayed at least three times each for a total of 17 991 spots. Total RNA from each treatment studied was converted to cDNA and labeled as described by Price et al (2005). Each treatment was labeled with each dye, removing effects on measurements caused by the individual dyes. The hybridized slides were scanned with a Perkin Elmer ScanArray Express Lite scanner (Perkin Elmer Life and Analytical Sciences Inc., Boston, Massachusetts). Spot intensity data were extracted from the images with UCSF-Spot (Jain et al 2002). The resulting spot-intensity data were analyzed with the mixed procedure in SAS (SAS v8, SAS Institute, Cary, North Carolina) as described by Price et al (2005). Briefly, least squares estimates of gene-specific treatment effects were obtained for each gene under each treatment. Differences between treatment effects (least squares estimates) for pairs of treatments can be considered as log2-transformed fold changes (Wolfinger et al 2001). Comparisons were made between cultures grown at 28 C and 37 C. The experimental design is provided (FIG. 1). The experiment was performed in three phases. In the first phase a dye-flip experiment was performed to compare expression levels at 28–37 C after 24 h. Next, a time course experiment was performed with cultures grown at 37 C for 8 h, 16 h and 24 h. Finally, the same time course was performed with cultures grown at 28 C. Data from these arrays were analyzed together using temperature as the treatment effect.

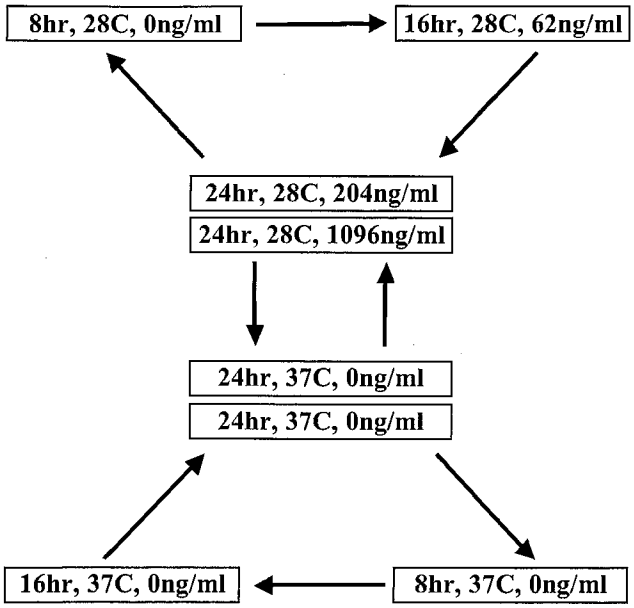

FIG. 1. Micro-array experimental design. Cultures of *A. flavus* were grown at either 28 C or 37 C for the period specified. The amount of aflatoxin produced in each culture is also indicated. RNA from each culture was isolated and cDNAs synthesized. cDNAs were labeled with either Cy3 or Cy5. Each arrow represents an array and depicts how the samples were labeled. cDNA derived from cultures at the tail of the arrow were labeled with Cy3 and those at the arrowhead were labeled with Cy5.

RESULTS

Aflatoxin production is regulated by temperature.—Time course experiments were performed to evaluate aflatoxin production in liquid cultures of *A. flavus* grown at various temperatures (FIG. 2A). In these experiments, particularly after 36 h of growth, aflatoxin production was the highest at 28 C. Decreasing amounts of aflatoxin were produced as temperature increased from 34 C to 37 C. Minimal amounts were produced at 37 C. This trend in aflatoxin production also occurred when cultures were grown on solid media. The results show that aflatoxin production peaked at 30 C and then decreased as *Aspergilli* were exposed to increasing temperatures (FIG. 2B).

Transfer of cultures from 28 C to 37 C stops aflatoxin biosynthesis.—A series of *A. flavus* 3357 cultures were grown in A&M media mother cultures at 37 C for 16 h and transferred to 28 C daughter cultures for varied amounts of time before being moved back to 37 C. The total postmother culture incubation time was 24 h regardless of temperature. Aflatoxin concentrations were determined by HPLC from two replicates that were pooled after 24 h incubation. The results (FIG. 3) indicated that aflatoxin biosynthesis begins at 28 C after 12 h. If cultures were allowed to grow at 28 C for the entire 24 h, 465 ng/mL aflatoxin was made. However transfer of cultures to 37 C resulted in no further significant accumulation of aflatoxin. Results were equivalent when repeating the experiments with *A. parasiticus* strain SU-1 (data not shown). In a separate experiment daughter cultures were grown at 28 C for 24 h and

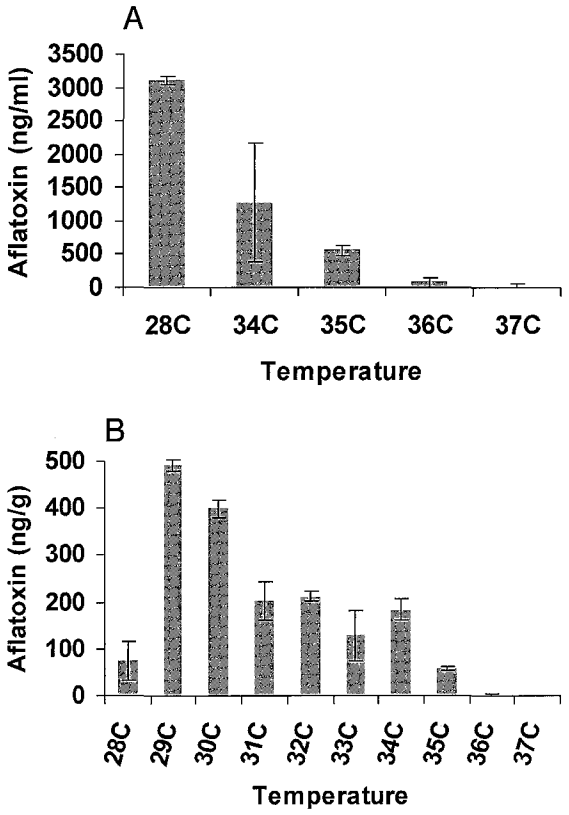

FIG. 2. Effect of temperature on aflatoxin production in liquid and solid media A. 1×10^6 spores/mL were seeded into 100 mL A&M medium and incubated at selected temperatures. The medium was sampled at 48 h and aflatoxin concentrations were determined by LC/MS. B. A total of 1×10^6 spores were plated on PDA medium and incubated at different temperatures. The medium was sampled at 24 h and aflatoxin concentrations were determined by HPLC.

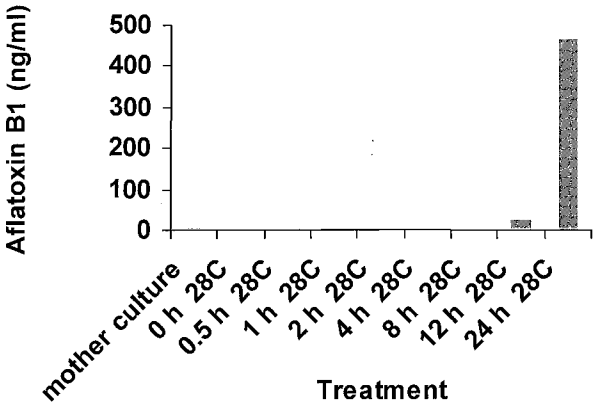

FIG. 3. Temperature shift. Cultures of *A. flavus* were grown in A&M medium at 37 C and moved to 28 C for indicated times before being returned to 37 C.

then placed at 37 C. Aflatoxin concentrations were determined at 0 h, 0.5 h, 1 h, 2 h, 4 h, 8 h and 24 h after transfer. The results show that no additional aflatoxin was made after transfer to 37 C (data not shown).

The transcription profile is different at 28–37 C.—Cultures were grown in A&M media for 8 h, 16 h and 24 h at 28 C and 37 C. Micro-array experiments were performed according to the design shown (FIG. 1). Only cultures grown for 16 h and 24 h at 28 C produced aflatoxin. A two-stage ANOVA approach was used to analyze the data (Wolfinger et al 2001). In this analysis temperature was the only treatment considered. Differences in least squares estimates of transcription levels were ranked according to p-value. A listing of the top 20 differentially expressed genes between the two temperatures are provided (TABLE I). Included in this list are three genes from the aflatoxin biosynthetic cluster, which were spotted as controls (known to be expressed during aflatoxin production). Three additional genes were also from the aflatoxin cluster. These were norsolorinic acid reductase, O-methyltransferase B and the aflatoxin polyketide synthase (also spotted as a control).

A total of 144 genes were differentially expressed ($P < 0.001$) between the two temperatures. Of these 103 were more highly expressed at 28 C. Among the genes more highly expressed at 28 C, approximately 25% were involved in secondary metabolism and about 30% were classified as hypothetical. Genes encoding a catalase and superoxide dismutase were among those more highly expressed at 37 C. These two genes also were found to be induced at 37 C in *Cryptococcus neoformans* (Kraus et al 2004).

Aflatoxin biosynthetic genes are more highly expressed at 28 C.—The least square means estimates for the aflatoxin biosynthetic cluster genes are illustrated (FIG. 4). Most aflatoxin genes were more highly expressed at 28 C relative to 37 C. However *aflR* and *aflS* did not follow this pattern. *AflR* and *aflS* showed about equal expression at both temperatures.

Quantitative PCR is consistent with micro-array data.—We used quantitative PCR and determined expression levels from cultures grown at 28 C, 35 C and 37 C for *aflR*, *aflR* antisense, *aflS* and *aflP*. As shown (TABLE II) levels of *aflS*, *aflR* and *aflR* antisense were relatively constant across each temperature tested. However the aflatoxin biosynthetic gene *aflP* was significantly more highly expressed at 28 C. There was also some expression of *aflP* at 35 C but no detectable expression at 37 C.

TABLE I. Top 20 differentially expressed genes between 28 C and 37 C

Aflatoxin genes	Higher at 28 C	Higher at 37 C
aflK (*vbs*)	NAFDA24TV	NAGAX81TV
aflY (*hypA*)	NAGDK09TV	NAFDI75TV
aflC (*pksA*)	NAGAH49TV	NAFCH04TV
NAFER13TV[a]	NAFCH39TV	NAGDF01TV
NAFBL32TV	NAFDO13TV	
NAFDL76TV	NAFFI87TV	
	NAFBW18TV	
	NAFAK20TV	
	NAGEF82TV	
	NAFAI15TV	

[a] Clone IDs were obtained from the TIGR website (http://www.tigr.org/tigr-scripts/tgi/T_reports.cgi?species=a_flavus).

DISCUSSION

The ecological significance of aflatoxin biosynthesis to *Aspergillus flavus* is unknown. Production of this secondary metabolite presumably contributes to the competitiveness of the fungus (Wilkinson et al 2004) and a large body of information shows that aflatoxin production can be induced by several substrates and environmental conditions. A better understanding of environmental regulation of this toxin may provide information regarding its role in the ecology of the fungus and lead to new approaches to reduce aflatoxin contamination of food.

In this study we focused on temperature as a modulator of aflatoxin production because it has one of the most striking effects of any environmental factor yet examined (Price et al 2005). While *Aspergillus flavus* grows over a wide range of temperatures in culture, its optimum temperature for growth is 37 C. To our surprise essentially no aflatoxin is produced at this temperature. Because temperature can have broad effects on fungi, several factors could account for reduced aflatoxin production at higher temperatures including changes in the metabolite partitioning, energy status of the cell or a direct effect of temperature on transcriptional regulatory circuits. We took advantage of a 5002

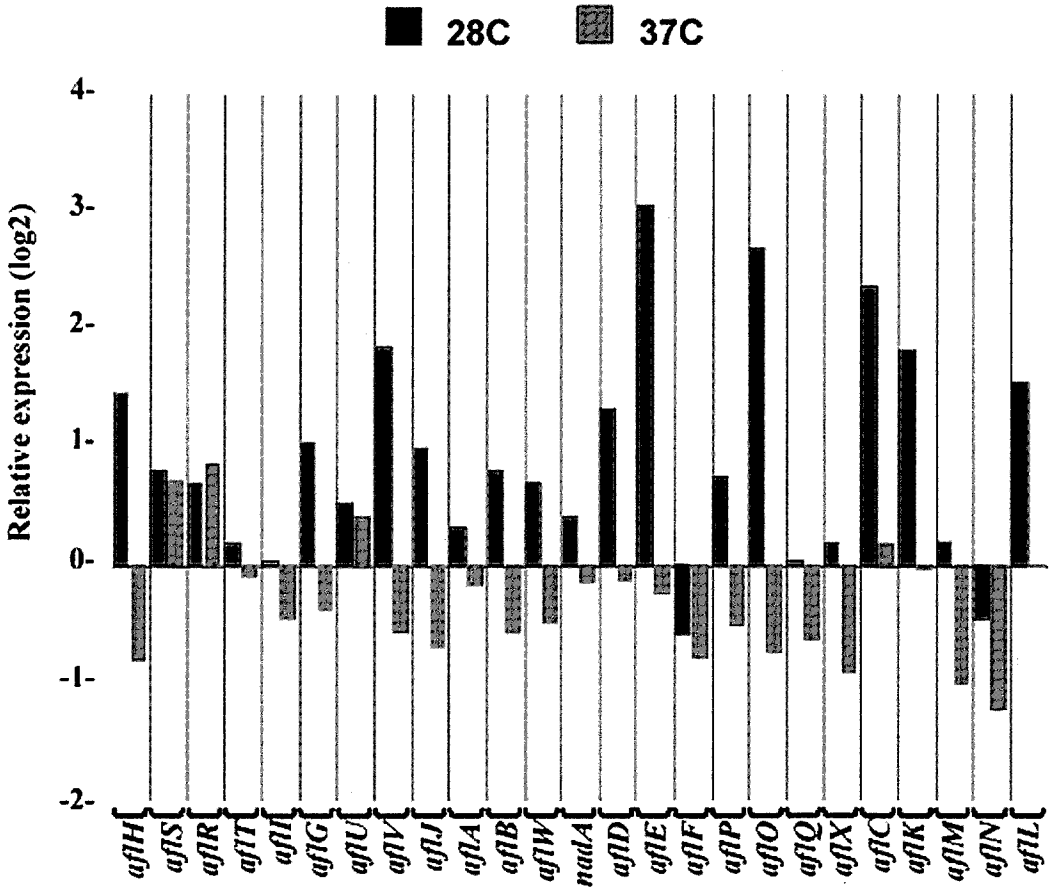

FIG. 4. Expression levels of aflatoxin pathway genes. Least square means estimates of expression levels obtained from the mixed-model analysis of the micro-array data were plotted for genes within the aflatoxin biosynthetic cluster. The analysis compared expression levels from cultures grown at 28 C and 37 C.

TABLE II. Fold gene induction

	aflS	*aflR*	*aflRas*	*aflP*
28 C	1.06	1.08	0.83	64.4
35 C	1.03	0.97	1.12	2.79
37 C	0.92	0.94	1.07	0

Fold induction measured relative to mean expression across a gene.

element DNA micro-array to better characterize the effect of temperature on gene transcription.

We observed the differential expression of 144 genes as temperature was increased from 28 C to 37 C. Of interest, most of these genes (103) were expressed more highly at 28 C. Approximately 25% of these genes are involved in secondary metabolism including aflatoxin biosynthesis (FIG. 4). Our study shows that temperature, directly or indirectly, affects the transcription of genes for secondary metabolism. These data are consistent with the transcription profiles reported for *aflP* (Liu and Chu 1998) and *aflC* (*pksA*) (Feng and Leonard 1995).

Even though little to no aflatoxin is produced at 37 C, we observed a low level of gene transcription for some of the pathway genes. This is probably due to basal transcription levels for these genes because aflatoxin pathway gene expression at 37 C followed a similar profile to that previously observed in an *aflR* deletion mutant (Price et al. 2006). We also were interested in learning if naturally occurring antisense of *aflR* discovered several years ago (Woloshuk et al 1994) played any role in the temperature response. Expression of this antisense along with other pathway genes is shown (TABLE II). The results of the quantitative PCR showed that levels of *aflS*, *aflR* and *aflR* antisense were relatively constant across each temperature tested. In contrast, the aflatoxin biosynthetic gene *aflP* was significantly more highly expressed at 28 C, with some expression at 35 C but no detectable expression at 37 C. These data obtained from quantitative PCR were consistent with those obtained from micro-array studies.

Our data argue that the failure of *A. flavus* to produce aflatoxin at 37 C is not due to the effect of temperature on the transcription of the pathway regulatory genes because transcript levels of *aflR* and *aflS* did not change significantly between 28 C and 37 C. One explanation for the temperature effect might be that less AFLR is produced at 37 C. Liu and Chu (1998) reported a lower concentration of AFLR at 37 C compared to 29 C. Another possibility is that AFLR is nonfunctional at higher temperatures. It is known that phosphorylation of AFLR interferes with the regulatory protein's activity because it may prevent the movement of AFLR into the nucleus (Shimizu et al 2003). Another possibility is that at elevated temperature, AFLS and AFLR are unable to interact; Chang (2003) has shown that AFLR and AFLS interact and together regulate transcription of the aflatoxin biosynthetic pathway. Additional studies are needed to determine the effect of temperature on AFLR and possibly on AFLS.

It is possible that other factors in addition to the nonfunctionality of AFLR affect aflatoxin production at elevated temperatures. For example the temperature response could be due to a modification of one or more of the pathway enzymes. This seems likely because aflatoxin production was greatly diminished in an aflatoxin producing culture after transferring from 28 C to 37 C (FIG. 3). At 28 C all of the necessary aflatoxin biosynthetic enzymes already had been made and were functioning to produce aflatoxin. After the shift to 37 C, production virtually ceased.

Our observations cannot rule out a direct effect of temperature on metabolic pathways that support aflatoxin biosynthesis. However it seems unlikely that an effect on these pathways would lead to such a rapid cessation of aflatoxin biosynthesis in cultures moved from 28 to 37 C. We found that the transfer to 37 C of aflatoxin producing cultures resulted in the almost immediate inhibition of aflatoxin synthesis. Others also have observed decreases in aflatoxin production at these temperatures (Schindler et al 1967, Schroeder and Hein 1967) or when cultures were exposed to elevated temperatures for short periods (Schroeder and Hein 1968).

Another interesting observation from this study is that the expression levels of *aflR* and *aflS* are relatively constant at both temperatures. This argues that the two genes may be transcriptionally coregulated. Perhaps they are both regulated by LAEA as has been proposed by Bok and Keller (2004). It also has been reported that AFLR does not transcriptionally regulate *aflS* or vice versa (Chang 2003).

In summary we have shown in this study that temperature affects aflatoxin production and the transcriptional profile of *A. flavus*. Transfer of an aflatoxin producing culture from 28 C to 37 C quickly turns off aflatoxin biosynthesis. The speed by which this occurs suggests that one or more of the pathway enzymes are posttranslationally regulated and are nonfunctional at 37 C. There is also a transcriptional component to temperature regulation. A larger number of genes are more highly expressed at 28 C relative to 37 C. We focused on the aflatoxin cluster genes and demonstrated a significant reduction in transcription at 37 C compared to 28 C. Although transcripts (and presumably protein) for the transcriptional regulator, *aflR*, and *aflS* are present at

37 C, the function of AFLR is inhibited at this temperature. We propose that one or both of these proteins may be nonfunctional at elevated temperatures in *A. flavus*.

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